Determination of antioxidant activity of phenolic compounds of *Thymus transcapicus* by high performance Liquid Chromatography

Maryam Sardarodiyan*, Mozhgan Nasiri Shahri**, Roya Amiri Qandashtani**, Ramezan Ali Mahian** and Elham Mahdian**

*Young Researchers and Elite Club, Quchan Branch, Islamic Azad University, Quchan, (Iran),
**Department of Food Science and Technology, Quchan Branch, Islamic Azad University,
Quchan, Iran

E-mail: sardarodiyan_5@yahoo.com

Abstract

*Thymus transcapicus* is a member of the genus *Thymus*. It is native to Iran with limited distribution in the Northeast. The *Thymus* is a traditional medicinal plant which is also used as a spice and aromatic plant in different industries. Resources survey showed that there is no report of antioxidant activity of *Thymus transcapicus*; therefore, this report can be considered as the first report on this subject. The main purpose of this study is to evaluate the antioxidant properties, total phenolic content of *Thymus transcapicus*. The determination has been done on three different extracts methanolic, dichloromethane and ethyl acetate. The methanolic fraction showed the highest antioxidant activity by DPPH-HPLC method, compared to dichloromethane and ethyl acetate. The total phenolic content was determined by the Folin-Ciocalteu method. The methanolic extract has been found to be rich in phenolic compound.

Keywords: *Thymus transcapicus*, methanolic, dichloromethane, ethyl acetate, and DPPH-HPLC

Introduction

With the increasing aging of the world’s population and people’s lifestyle, the occurrence of oxidative stress in cells, and therefore, the production of reactive species of oxygen (ROS) is also growing (Carocho and Ferreira, 2013). Oxidative stress, defined as “an imbalance between oxidants and antioxidants in favour of the oxidants, which is potentially cause damage” (Sies, 1997), is associated with higher risks of many diseases, including diabetes mellitus, hypertension, obesity and inflammation (Hopps et al., 2010).

Reactive oxygen species (ROS) such as superoxide anions (-OOH), hydrogen peroxide (H₂O₂) and hydroxyl radicals (.OH) induce Oxidative stress. ROS are generated as by-products of aerobic respiration and metabolism” (Al-Gubory et al., 2010), and modulated by antioxidant enzymes and non-enzymatic scavengers (Taleb-Senouci et al., 2009). Natural antioxidants are generally obtained from plants and vegetables, which are needed to counteract the damage of ROS to cells.

Flavonoids are a large group of plant polyphenol secondary metabolites and can be found widely in the leaves, seeds, bark and plants flowers. It is already well recognised that flavonoids possess anti-tumoral, antiischemic, antiallergic, anti-inflammatory and antibacterial activities. Moreover, flavonoids show strong antioxidant capacities through scavenging oxygen free radicals.
radicals, promote anti-oxidase or inhibit oxidative enzymes (Harborne and Williams, 1992 and 2000).

Phenolic compounds are secondary plant metabolites that play a key role in the sensory and nutritional quality of fruits, vegetables and other plants (Ignat et al., 2011). Antioxidant activities are known to increase proportionally to the polyphenol content, mainly due to their redox properties (Rasineni et al., 2008). Among the diverse roles of polyphenols, they protect cell constituents against destructive oxidative damage, thus limiting the risk of various degenerative diseases associated with oxidative stress and thus tending to be potent free radical scavengers. Their ability to act as antioxidants is due to their chemical structure and ability to donate/accept electrons, thus delocalizing the unpaired electron within the aromatic structure (Ross and Kasum, 2002).

*Thymus transcapicus* is a member of the genus Thymus. It is native in Iran (Mozaffarian, 1996) with limited distribution in the Northeast and Turkmenistan and also this species is a restricted distribution in the Northeast of Iran, in addition grows in high altitudes almost from 1700 to 2800 m (Rechinger, 1982 and Borisova et al., 1997). The genus *Thymus* L. (Lamiaceae), is an aromatic and medicinal plant, includes numerous species with quite different botanical characteristics. Thyme oil is used for the treatment of bacterial and fungal infections, in mouthwash as an expectorant (Tabrizi et al., 2010). The essential oil of *T. vulgaris* is used as antibacterial, antifungal, antioxidant, antiseptic in acne and skin problems, carminative, diuretic, expectorant and mental stimulant in aromatherapy (Price and Price. 1999). Thymol is the main component of many Thymus spp, which is known as an antiseptic agent and used for hookworm treatment (Evans, 1998). There are no literature reports on antioxidant activity of *Thymus transcapicus* and the main purpose of this study was to evaluate the antioxidant activity of *Thymus transcapicus* through DPPH-HPLC method and determination of the total phenolic content of it.

**Methods and Materials**

**Plant material**

The plant material was collected in May 2016 from North Khorasan Province Mountains in Iran. Then, the plant was identified and confirmed by Natural Products and Medicinal Plants Research Centre, North Khorasan University of Medical Sciences (Iran) and Voucher specimen (No: MP 32/4) was deposited in the herbarium of the Natural Products and Medicinal Plants Research Centre.

**Chemicals and reagents**

The following chemicals were purchased: Methanol (Chromasolv, ≥99.9%, Sigma-Aldrich), dichloromethane (650463, Chromasolv, ≥99.9%, Sigma-Aldrich), ethyl acetate (439169, Chromasolv, ≥99.8%, Sigma-Aldrich), Folin-Ciocalteau reagent (F9252, Sigma-Aldrich), Na2CO3 (451614, anhydrous powder, 99.999%, Sigma-Aldrich), Gallic acid (91215, Fluka), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (257621, Sigma-Aldrich), ascorbic acid (A130000, European Pharmacopoeia (EP) Reference Standard, Fluka), BHT (W21 8405 ≥ 99, Sigma-Aldrich), distilled water (115333, Water for chromatography LiChrosolv).

**High Performance Liquid Chromatography condition**

High performance liquid chromatography (HPLC) was run on a LC-6AD pump (Shimadzu, Kyoto, Japan) connected to a SPD-M20A Diode Array detector (Shimadzu) and the column was analytical Shim-pack ODS-A, 4.6 × 250 mm, 5 μm (Shimadzu, Japan).

**Preparation of plant extracts**

The aerial parts of the plant were dried under shade at room temperature and then cut into small pieces. About 100 g of sample was separately left in
three different solvents (methanol, dichloromethane and ethyl acetate) at 25°C. Each solvent was allowed to remain in contact with plant material for 2 days, and replaced with fresh solvent four times. Removal of solvents under vacuum at 40 °C gave the crude extracts (Prachayasittikul et al., 2008).

**Determination of total phenolic content**

Analysis of total phenolic content was conducted using a previously described protocol (Ku et al., 2010). Ten-microliter sample extracts were added to 0.2 N Folin-Ciocalteu’s phenol reagent (100 mL) in 96-well plates. After 3 min, 90 μL of a saturated sodium carbonate solution was added to the mixture and subsequently incubated at room temperature for 1 h. The resulting absorbance of the mixture was measured at 630 nm using a BioTek EL 808 microplate reader (Power Wave XS; Biotek Instruments Inc., Winooski, VT). The total phenolic content was calculated on the basis of a standard curve using gallic acid (concentration range 31.25 to 500 mg•mL⁻¹). Results are expressed in milligrams of gallic acid equivalents per 100 g of dried broccoli. Three biologically replicated (block) samples were assayed with three analytical replications each.

**Evaluation of antioxidant activity HPLC analysis for DPPH radical scavenging**

The antioxidant activities of the extracts were described by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity of extracts (Chandra-sekar et al., 2006).

Fresh DPPH stock solution at a concentration of 2.5 mM was prepared. Then, 100 μl of plant extract at several concentrations (125-500 g. ml⁻¹) was added to 100 μl of DPPH solution (final concentration 250 M. 200 μl⁻¹). The mixture was vortexed for a few seconds and left in the dark for 20 min at room temperature. After that, 20 μl of the sample was injected to HPLC. The blank was prepared by adding 100 μl of methanol to 100 μl of the DPPH stock solution. Analyses were carried out using a Reversed-phase (RP) column (250 mm × 4.6 mm, 5 μm). Isocratic elution was carried out with methanol/water (80:20, v/v) at a flow rate of 1 ml.min⁻¹. The DPPH peaks were monitored at 517 nm and 325 nm. The difference in the reduction of DPPH peak area (PA) between the blank and the sample was used for determining the percent radical scavenging activity of the sample by using the following formula 1:

\[
\text{Radical scavenging} (\%) = \frac{\text{PA blank} - \text{PA sample}}{\text{PA blank}} \times 100
\]

Ascorbic acid and butylated hydroxy toluene (BHT) were used as positive controls. Absorbance inhibition (AI) and 50% effective concentration (EC₅₀) values were calculated using Graph Pad Prism software, version 5.01 (Graph Pad Software Inc., San Diego CA, USA) (Chen et al., 2013).

**Results and Discussion**

The antioxidant activity is generally attributed to phenolic compounds in plant extracts (Babbar et al., 2011). The redox properties of phenolic compounds make them behave as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans, 1996). Solvent polarity plays a remarkable role in phenolic compounds extraction and methanol is an efficient solvent in their extraction (Hernandez-Hernandez, 2009).

In this study three different solvents with different polarities were used. As shown in Table 1 the yield of methanolic extracts was the highest (2.92%) among these three solvents due to its high plarity compared to dichloromethane (1.78%) and ethyl acetate (1.58%).

The total phenolic content of these three extracts were determined by Gallic acid (mg) per dried extracts
Table 1. Extraction Yield, Total Phenolic Content and Antioxidant Activity of Thymus transcapicus

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Extraction yield (%)</th>
<th>Total phenolic (Gallic acid equivalents mg·g⁻¹ of dry extract)</th>
<th>IC₅₀ via HPLC-DPPH (mg·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
<td>1.78</td>
<td>0.097</td>
<td>6.57</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.58</td>
<td>0.467</td>
<td>1.41</td>
</tr>
<tr>
<td>Methanol</td>
<td>2.92</td>
<td>1.142</td>
<td>0.563</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>---</td>
<td>---</td>
<td>0.00097</td>
</tr>
<tr>
<td>BHT</td>
<td>---</td>
<td>---</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Fig. 1. HPLC-DPPH chromatograms of methanolic extract in different concentrations

Fig. 2. DPPH-HPLC chromatograms for dichloromethane extract in different concentrations
As shown in figures 1,2 and 3 with increasing in sample concentration the peak areas, remarkably decreased after spiking the DPPH solution. The sample peak areas are shown in Table 2.

Figure 4 shows %DPPH inhibition with concentration. Results were reported as IC$_{50}$, which is defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals and in this method, it is well-known that the lower IC$_{50}$ has the higher antiradical activity. IC$_{50}$ values of extracts are shown in Table - 1.

Figure 4 shows that as the concentration of extract increased the % DPPH inhibition increased. The maximum concentration of methanolic extract (4 mg. ml$^{-1}$) was able to inhibit 90.45% of DPPH. For ethyl acetate the %DPPH inhibition of the maximum concentration (8 mg.ml$^{-1}$) is 91.06% which is lower
Table 2: Sample peak areas for different solvents in different concentrations

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Blank 0.125 mg. ml⁻¹</th>
<th>0.25 mg. ml⁻¹</th>
<th>0.5 mg. ml⁻¹</th>
<th>1 mg. ml⁻¹</th>
<th>2 mg. ml⁻¹</th>
<th>4 mg. ml⁻¹</th>
<th>8 mg. ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloro methane</td>
<td>12144538</td>
<td>10890850</td>
<td>11150728</td>
<td>10205555</td>
<td>10220775</td>
<td>9579846</td>
<td>7782383</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>12144538</td>
<td>11021477</td>
<td>10769242</td>
<td>10752576</td>
<td>6445323</td>
<td>5861567</td>
<td>2902379</td>
</tr>
<tr>
<td>Methanol</td>
<td>12144538</td>
<td>11072816</td>
<td>7750894</td>
<td>6707869</td>
<td>4405944</td>
<td>1335895</td>
<td>1159320</td>
</tr>
</tbody>
</table>

Fig. 4. % DPPH inhibition in different concentrations

In this study, Ascorbic acid had the highest radical scavenging activities than Thymus transcapicus extracts. Among these extracts, methanolic extract showed the highest antioxidant activity. Moreover, this extract had the highest amount of phenolic content (IC₅₀ = 0.563 mg. ml⁻¹) in comparison with ethyl acetate and dichloromethane extracts. The difference between the total phenolic content of three extracts leads to different antioxidant activity (Singh et al., 2007).

Conclusion

The result of the present study showed that the extract which had the highest amount of phenolic compound (methanol extract), exhibited the greatest antioxidant activity and which was responsible for the antioxidant activity for this plant. The findings of this study support this view that some medicinal plants are promising sources of potential antioxidants and could be used as preventive agents for some diseases.

Acknowledgements

The authors thank the members of the Islamic the Azad University, Quchan Branch, Iran, for their technical assistance.

References


Corresponding Author : Maryam Sardarodiyan, Young Researchers and Elite Club, Quchan Branch, Islamic Azad University, Quchan, (Iran), E-mail: sardarodiyan_5@yahoo.com, ©2017, IJALS. All Rights Reserved.
https://doi.org/10.26627/IJALS/2017/10.02.0012